PATENT
Attorney Docket No. **FORS-01756**

#19
4-22-99

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Mary Ann D. Brow *et al.*
Serial No.: 08/520,946
Filed: 08/30/95
Entitled: **Rapid Detection And Identification Of Pathogens**

Group No.: 1636
Examiner: W. Sandals

TRANSMITTAL OF APPEAL BRIEF (PATENT APPLICATION - 37 CFR § 192)

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: February 22, 1999 By: Marlene Garitano
Marlene Garitano

Sir:

1. Transmitted herewith, in triplicate, is the APPEAL BRIEF in this application, with respect to the Notice of Appeal filed on **December 21, 1998**.

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2. **STATUS OF APPLICANT**

This application is on behalf of

a small entity.

A verified statement has already been filed.

3. **FEE FOR FILING APPEAL BRIEF**

Pursuant to 37 CFR § 1.17(g), the fee for filing the Appeal Brief is:

Fee for Filing Appeal Brief $150.00

4. **EXTENSION OF TERM**

The proceedings herein are for a patent application and the provisions of 37 CFR § 1.136 apply.

Applicant believes that no extension of term is required. However, this conditional petition is being made to provide for the possibility that applicant has inadvertently overlooked the need for a petition and fee for extension of time.

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5. TOTAL FEE DUE

The total fee due is:

Appeal brief fee $150.00

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6. FEE PAYMENT

Attached is a check for **$150.00**.

7. FEE DEFICIENCY

If any additional fee is required, charge Account No. **08-1290**.

Dated: February 22, 1999

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APPELLANTS' BRIEF
APPEAL NO.:

ATTN: Board of Patent Appeals and Interferences
Commissioner for Patents and Trademarks
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 CFR § 1.8(a)

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to the: Assistant Commissioner of Patents, Washington, D.C. 20231, on February 22, 1999.

By: Marlene E. Garitano
Marlene E. Garitano

Sir:

This Brief is in furtherance of the Notice of Appeal.

The fees required under § 1.17(h) and any required Petition for Extension of Time for filing this Brief and fees therefore are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

This Brief is transmitted in triplicate. [37 CFR § 1.192(a).]

This Brief contains these items under the following headings and in the order set forth below [37 CFR § 1.192(c)]:

I. REAL PARTY IN INTEREST

The real party in interest is the assignee of record, Third Wave Technologies, Inc., a corporation of the State of Wisconsin, 1250 Cloverleaf Drive, Monrovia, California 91016.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences known to Appellant or to Appellant's legal representative.

III. STATUS OF CLAIMS

The present application is a Continued Prosecution Application of Application Serial Number 08/520,946, filed 08/30/95. Claims 1-44 were filed in the original application. During prosecution of the application, Claims 2 and 30 were cancelled without prejudice and Claims 45-54 were added. Claims 1, 3-29, and 31-54 have been rejected by the Examiner in the latest Office Action. Therefore, Claims 1, 3-29, and 31-54 are pending in this Appeal. Appellant appeals the Final Rejection dated 8/31/98.

The Claims, as they now stand, are set forth in Appendix A (attached at Tab A).

IV. STATUS OF AMENDMENTS

Appellants provided amendments to the specification and claims in a Response to the Final Office Action. The amendments have not been entered, as the Examiner alleges that the amendments are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal. With respect to the amendments to the Specification, the Examiner has requested a substitute specification because the number of

amendments constitutes an unreasonable burden. Appellants will provide a substitute specification under 37 CFR 1.125(a) should the claims be determined allowable. With respect to the amendments to the Claims, Appellants request that said amendments be entered with this filing of an Appeal Brief. A copy of the amendments, as set forth in Appellants' Response to Final Office Action are set forth in Appendix B (attached at Tab B).

V. SUMMARY OF INVENTION

The present invention relates to methods and compositions for treating nucleic acid, and in particular, methods and compositions for detection and characterization of nucleic acid sequences and sequence changes in microbial gene sequences. The present invention provides means for cleaving a nucleic acid cleavage structure in a site-specific manner. In one embodiment, the means for cleaving is an enzyme capable of cleaving cleavage structures on a nucleic acid substrate, forming the basis of a novel method of detection of specific nucleic acid sequences. As claimed, the cleavage structures comprise intra-strand secondary structure. Thus, the cleavage events are based on the structure of the nucleic acid target rather than the sequence. The present invention contemplates use of the novel detection method for, among other uses, clinical diagnostic purposes, including but not limited to detection and identification of pathogenic organisms.

In one embodiment, the present invention contemplates a DNA sequence encoding a DNA polymerase altered in sequence (*i.e.*, a "mutant" DNA polymerase) relative to the native sequence such that it exhibits altered DNA synthetic activity from that of the native (*i.e.*, "wild type") DNA polymerase. With regard to the polymerase, a complete absence of synthesis is not required; it is desired that cleavage reactions occur in the absence of

polymerase activity at a level where it interferes with the method. It is preferred that the encoded DNA polymerase is altered such that it exhibits reduced synthetic activity from that of the native DNA polymerase. In this manner, the enzymes of the invention are nucleases and are capable of cleaving nucleic acids in a structure-specific manner. Importantly, the nucleases of the present invention are capable of cleaving cleavage structures to create discrete cleavage products.

The present invention contemplates nucleases from a variety of sources, including nucleases that are thermostable. Thermostable nucleases are contemplated as particularly useful, as they are capable of operating at temperatures where nucleic acid hybridization is extremely specific, allowing for allele-specific detection (including single-base mismatches). In one embodiment, the thermostable 5' nucleases are selected from the group consisting of altered polymerases derived from the native polymerases of various *Thermus* species, including, but not limited to *Thermus aquaticus*, *Thermus flavus* and *Thermus thermophilus*.

The present invention utilizes such enzymes in methods for detection and characterization of nucleic acid sequences and sequence changes. The present invention relates to means for cleaving a nucleic acid cleavage structure in a site-specific manner. Nuclease activity is used to screen for known and unknown mutations, including single base changes, in nucleic acids.

In one embodiment, the present invention contemplates a process or method for identifying strains of microorganisms comprising the steps of providing a cleavage means and a nucleic acid substrate containing sequences derived from one or more microorganism; treating the nucleic acid substrate under conditions such that the substrate forms one or more cleavage structures; and reacting the cleavage means with the cleavage structures so that one

or more cleavage products are produced. In one embodiment of this invention, the cleavage means is an enzyme. In one preferred embodiment, the enzyme is a nuclease. In an alternative preferred embodiment, the nuclease is selected from the group consisting of Cleavase™ BN nuclease, *Thermus aquaticus* DNA polymerase, *Thermus thermophilus* DNA polymerase, *Escherichia coli* Exo III, and the *Saccharomyces cerevisiae* Rad1/Rad10 complex. It is also contemplated that the enzyme may have a portion of its amino acid sequence that is homologous to a portion of the amino acid sequence of a thermostable DNA polymerase derived from a eubacterial thermophile, the latter being selected from the group consisting of *Thermus aquaticus*, *Thermus flavus* and *Thermus thermophilus*.

It is contemplated that the nucleic acid substrate comprise a nucleotide analog, including but not limited to the group comprising 7-deaza-dATP, 7-deaza-dGTP and dUTP. In one embodiment, the nucleic acid substrate is substantially single-stranded. It is not intended that the nucleic acid substrate be limited to any particular form, indeed, it is contemplated that the nucleic acid substrate is single stranded or double-stranded RNA or DNA.

In one embodiment of the present invention, the treating step comprises rendering double-stranded nucleic acid substantially single-stranded, and exposing the single-stranded nucleic acid to conditions such that the single-stranded nucleic acid assumes a secondary or characteristic folded structure. In one preferred embodiment, double-stranded nucleic acid is rendered substantially single-stranded by increased temperature.

In an alternative embodiment, the method of the present invention further comprises the step of detecting one or more cleavage products.

It is contemplated that the microorganism(s) of the present invention be selected from a variety of microorganisms. It is not intended that the present invention be limited to any particular type of microorganism. Rather, it is intended that the present invention be used with organisms including, but not limited to, bacteria, fungi, protozoa, ciliates, and viruses. It is not intended that the microorganisms be limited to a particular genus, species, strain, or serotype. Indeed, it is contemplated that the bacteria be selected from the group including, but not limited to members of the genera *Campylobacter, Escherichia, Mycobacterium, Salmonella, Shigella,* and *Staphylococcus*. In one preferred embodiment, the microorganism(s) comprise strains of multi-drug resistant *Mycobacterium tuberculosis*. It is also contemplated that the present invention be used with viruses, including but not limited to hepatitis C virus and simian immunodeficiency virus.

Another embodiment of the present invention contemplates a method for detecting and identifying strains of microorganisms, comprising the steps of extracting nucleic acid from a sample suspected of containing one or more microorganisms; and contacting the extracted nucleic acid with a cleavage means under conditions such that the extracted nucleic acid forms one or more secondary structures, and the cleavage means cleaves the secondary structures to produce one or more cleavage products.

In one embodiment, the method further comprises the step of separating the cleavage products. In yet another embodiment, the method further comprises the step of detecting the cleavage products.

In one preferred embodiment, the present invention further comprises comparing the detected cleavage products generated from cleavage of the extracted nucleic acid isolated from the sample with separated cleavage products generated by cleavage of nucleic acids derived

from one or more reference microorganisms. In such a case, the sequence of the nucleic acids from one or more reference microorganisms may be related but different (*e.g.*, a wild type control for a mutant sequence or a known or previously characterized mutant sequence).

In an alternative preferred embodiment, the present invention further comprises the step of isolating a polymorphic locus from the extracted nucleic acid after the extraction step, so as to generate a nucleic acid substrate, wherein the substrate is contacted with the cleavage means. In one embodiment, the isolation of a polymorphic locus is accomplished by polymerase chain reaction (PCR) amplification. In an alternate embodiment, the polymerase chain reaction is conducted in the presence of a nucleotide analog, including but not limited to the group comprising 7-deaza-dATP, 7-deaza-dGTP and dUTP. It is contemplated that the polymerase chain reaction amplification will employ oligonucleotide primers matching or complementary to consensus gene sequences derived from the polymorphic locus. In one embodiment, the polymorphic locus comprises a ribosomal RNA gene. In a particularly preferred embodiment, the ribosomal RNA gene is a 16S ribosomal RNA gene.

In yet another embodiment, the present invention contemplates a method for treating nucleic acid comprising an oligonucleotide containing microbial gene sequences, comprising providing a cleavage means in a solution containing manganese and nucleic acid substrate containing microbial gene sequences; treating the nucleic acid substrate with increased temperature such that the substrate is substantially single-stranded; reducing the temperature under conditions such that the single-stranded substrate forms one or more cleavage structures; reacting the cleavage means with the cleavage structures so that one or more cleavage products are produced; and detecting the one or more cleavage products produced by the method.

The present invention also contemplates a process for creating a record reference library of genetic fingerprints characteristic (*i.e.*, diagnostic) of one or more alleles of the various microorganisms, comprising the steps of providing a cleavage means and nucleic acid substrate derived from microbial gene sequences; contacting the nucleic acid substrate with a cleavage means under conditions such that the extracted nucleic acid forms one or more secondary structures and the cleavage means cleaves the secondary structures, resulting in the generation of multiple cleavage products; separating the multiple cleavage products; and maintaining a testable record reference of the separated cleavage products.

By the term "genetic fingerprint" it is meant that changes in the sequence of the nucleic acid (*e.g.*, a deletion, insertion or a single point substitution) alter the structures formed, thus changing the banding pattern (*i.e.*, the "fingerprint" or "bar code") to reflect the difference in the sequence, allowing rapid detection and identification of variants.

The methods of the present invention allow for simultaneous analysis of both strands (*e.g.*, the sense and antisense strands) and are ideal for high-level multiplexing. The products produced are amenable to qualitative, quantitative and positional analysis. The methods may be automated and may be practiced in solution or in the solid phase (*e.g.*, on a solid support). The methods are powerful in that they allow for analysis of longer fragments of nucleic acid than current methodologies.

VI. ISSUES

There is one issue involved in the present appeal: whether Claims 1, 3-29, and 31-54 are obvious under Lyamichev *et al.* (Science 260:778-783), in view of Young (U.S. Patent

No. 5,422,242), Seela and Roling (Nucl. Acids Res. 20:55-61), and Young *et al.* (J. Clin. Microbiol. 31:882-886).

VII. GROUPING OF CLAIMS

Each claim stands alone. Each claim has separate limitations and must be considered independently.

Independent Claim 1 specifies a method comprising providing an enzymatic cleavage means, a test nucleic acid substrate containing sequences derived from one or more microorganisms, and control cleavage products produced by cleavage of a reference nucleic acid derived from a microorganism; treating the test nucleic acid substrate under conditions such that the substrate forms one or more intra-strand secondary structures; reacting the cleavage means with the cleavage structures so that one or more test cleavage products are produced; and comparing the test cleavage products to the control cleavage products. The Claim 1 is not limited to a particular type of cleavage means.

Dependent Claim 3 specifies that the enzymatic cleavage means is a nuclease. Dependent Claim 4 specifies that the nuclease is selected from the group consisting of Cleavase™ BN nuclease, *Thermus aquaticus* DNA polymerase, *Thermus thermophilus* DNA polymerase, *Escherichia coli* Exo III, and the *Saccharomyces cerevisiae* Rad1/Rad10 complex. Dependent Claim 5 specifies that the test nucleic acid substrate comprises a nucleotide analog. Dependent Claim 6 specifies that the nucleotide analog is selected from the group comprising 7-deaza-dATP, 7-deaza-dGTP and dUTP. Dependent Claim 7 specifies that the test nucleic acid of step (a) is substantially single-stranded. Dependent Claim 8 specifies that the test nucleic acid is RNA. Dependent Claim 9 specifies that the test nucleic acid is DNA.

Dependent Claim 10 specifies that the nucleic acid of step (a) is double stranded. Dependent Claim 11 specifies that the treating step (b) in Claim 10 comprises rendering the double-stranded nucleic acid substantially single-stranded; and exposing the single-stranded nucleic acid to conditions such that the single-stranded nucleic acid has secondary structure. Dependent Claim 12 specifies that the double-stranded nucleic acid of Claim 11 is rendered substantially single-stranded by increased temperature. Dependent Claim 13 specifies that the method of Claim 1 further comprises the step of detecting the one or more cleavage products. Dependent Claim 14 specifies that the microorganism comprises bacteria. Dependent Claim 15 specifies that the bacteria are selected from the group comprising members of the genera *Campylobacter, Escherichia, Mycobacterium, Salmonella, Shigella* and *Staphylococcus.* Dependent Claim 16 specifies that the members of the genus *Mycobacterium* comprise strains of multi-drug resistant *Mycobacterium tuberculosis.* Dependent Claim 17 specifies that the microorganism comprises virus. Dependent Claim 18 specifies that the virus is selected from the group comprising hepatitis C virus and simian immunodeficiency virus.

Independent Claim 19 specifies a method for treating nucleic acid comprising extracting nucleic acid from a sample suspected of containing one or more microorganisms; and contacting the extracted nucleic acid with an enzymatic cleavage means under conditions such that the extracted nucleic acid forms one or more intra-strand secondary structures, and the cleavage means cleaves the intra-strand secondary structures to produce a plurality of cleavage products. Dependent Claim 20 specifies the method of Claim 19 further comprising the step of separating the cleavage products. Dependent Claim 21 specifies the method of Claim 19 further comprising the step of detecting said cleavage products. Dependent Claim 22 specifies the method of Claim 21 further comprising comparing the detected cleavage

products generated from cleavage of the extracted nucleic acid isolated from the sample with separated cleavage products generated by cleavage of nucleic acids derived from one or more reference microorganisms. Dependent Claim 23 specifies the method of Claim 19 further comprising the step of isolating a polymorphic locus from the extracted nucleic acid after the extraction of step (a), to generate a nucleic acid substrate wherein the substrate is contacted with the cleavage means of step (b). Dependent Claim 24 specifies that the isolation of a polymorphic locus is accomplished by polymerase chain reaction amplification. Dependent Claim 25 specifies that the polymerase chain reaction is conducted in the presence of a nucleotide analog. Dependent Claim 26 specifies that the nucleotide analog of Claim 25 is selected from the group comprising 7-deaza-dATP, 7-deaza-dGTP and dUTP. Dependent Claim 27 specifies that the polymerase chain reaction amplification employs oligonucleotide primers matching or complementary to consensus gene sequences derived from the polymorphic locus. Dependent Claim 28 specifies that the polymorphic locus comprises a ribosomal RNA gene. Dependent Claim 29 specifies that the ribosomal RNA gene of Claim 28 is a 16S ribosomal RNA gene. Dependent Claim 31 specifies that the enzymatic cleavage means is a nuclease. Dependent Claim 32 specifies that the nuclease is selected from the group consisting of Cleavase™ BN nuclease, *Thermus aquaticus* DNA polymerase, *Thermus thermophilus* DNA polymerase, *Escherichia coli* Exo III, and the *Saccharomyces cerevisiae* Rad1/Rad10 complex. Dependent Claim 33 specifies that the nucleic acid of step (a) in Claim 19 is substantially single-stranded. Dependent Claim 34 specifies that the nucleic acid is RNA. Dependent Claim 35 specifies that the nucleic acid is DNA. Dependent Claim 36 specifies that the nucleic acid of step (a) is double stranded. Dependent Claim 37 specifies that the treating of step (b) comprises rendering the double-stranded nucleic acid substantially

single-stranded; and exposing the single-stranded nucleic acid to conditions such that the single-stranded nucleic acid has secondary structure. Dependent Claim 38 specifies that the double-stranded nucleic acid of Claim 37 is rendered substantially single-stranded by increased temperature. Dependent Claim 39 specifies that the microorganism comprises bacteria. Dependent Claim 40 specifies that the bacteria are selected from the group comprising members of the genera *Campylobacter, Escherichia, Mycobacterium, Salmonella, Shigella* and *Staphylococcus*. Dependent Claim 41 specifies that the members of the genus *Mycobacterium* comprise strains of multi-drug resistant *Mycobacterium tuberculosis*. Dependent Claim 42 specifies that the microorganism comprises virus. Dependent Claim 43 specifies that the virus is selected from the group comprising hepatitis C virus and simian immunodeficiency virus.

Independent Claim 44 specifies a method comprising providing an enzymatic cleavage means in a solution comprising manganese and nucleic acid substrate containing microbial gene sequences; treating the nucleic acid substrate with increased temperature such that substantially single-stranded substrate is produced; reducing the temperature under conditions such that the single-stranded substrate forms one or more cleavage structures; reacting the cleavage means with the cleavage structures so that one or more cleavage products are produced; and detecting the one or more cleavage products. Dependent Claim 45 specifies that the enzymatic cleavage means is a nuclease. Dependent Claim 46 specifies that the nuclease is selected from the group consisting of Cleavase™ BN nuclease, *Thermus aquaticus* DNA polymerase, *Thermus thermophilus* DNA polymerase, *Escherichia coli* Exo III, and the *Saccharomyces cerevisiae* Rad1/Rad10 complex. Dependent Claim 47 specifies that the nucleic acid substrate comprises a nucleotide analog. Dependent Claim 48 specifies that the

nucleotide analog is selected from the group comprising 7-deaza-dATP, 7-deaza-dGTP and dUTP. Dependent Claim 44 specifies that the nucleic acid is selected from the group consisting of RNA, double stranded DNA and single stranded DNA. Dependent Claim 50 specifies that the microorganism comprises bacteria. Dependent Claim 51 specifies that the bacteria are selected from the group comprising members of the genera *Campylobacter, Escherichia, Mycobacterium, Salmonella, Shigella* and *Staphylococcus*. Dependent Claim 52 specifies that the members of the genus *Mycobacterium* comprise strains of multi-drug resistant *Mycobacterium tuberculosis*. Dependent Claim 53 specifies that the microorganism comprises virus. Dependent Claim 54 specifies that the virus is selected from the group comprising hepatitis C virus and simian immunodeficiency virus.

Because each of the independent Claims have different limitations, they do not stand or fall together. Rather, they need to be evaluated separately.

VIII. ARGUMENT

The Claimed invention is Nonobvious

Appellants assert that the Examiner has not met the burden of establishing a *prima facie* case of obviousness. A *prima facie* case of obviousness requires the Examiner to cite to references that (a) disclose all the elements of the claimed invention, (b) suggest or motivate one of skill in the art to combine or modify those elements to yield the claimed combination, and (c) provide a reasonable expectation of success should the claimed combination be carried

out.[1] Failure to establish **any one** of these three requirements precludes a finding of a *prima facie* case and, without more, entitles Appellants to allowance of the claims at issue. The cited art fails to establish *prima facie* obviousness because there is no motivation to combine the cited references. Furthermore, even if such a combination were made, the cited references do not teach or suggest every element of the presently claimed invention. Thus, Appellants assert that Claims 1, 3-29, and 31-54 are unobvious over the art cited by the Examiner.

In particular, none of the references cited by the Examiner (*i.e.*, Lyamichev *et al.*, Young, Seela and Roling, and Young *et al.*), alone, or in combination, teach or suggest characterization of sequence variation or any other applications by cleavage of intra-strand secondary structures. Indeed, as discussed below, Lyamichev *et al.*, Young, Seela and Roling, and Young *et al.* teach away from the use of intra-strand secondary structures as targets for cleavage means.

I. There is No Motivation to Combine the References

Obviousness cannot be established by combining the teaching of the prior art to produce the claimed invention, absent some teaching, suggestion or incentive supporting the combination.[2] Appellants assert that the cited references do not contain any suggestion for their combination.

The presently claimed invention provides methods for characterization of nucleic acid sequences and sequence changes using a cleavage means that recognizes **intrastand**

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[1] *See, e.g., Northern Telecom Inc. v. Datapoint Corp.*, 15 USPQ2d 1321, 1323 (Fed. Cir. 1990); and *In re Dow Chemical Co.*, 837 F.2d 469, 5 USPQ2d 1529 (Fed. Cir. 1988).

[2] *In re Geiger,* 2 USPQ 2d 1276, 1278 (Fed. Cir. 1987).

secondary structure (*i.e.*, folded nucleic acid structure).[3] This is in contrast to methods based on nucleic acid **sequence** (*e.g.*, PCR, Q-Beta replicase, Ligase Chain Reaction, Northern and Southern Blotting, and RNase Protection assays). As described in the specification (*See e.g.*, pages 1-13), there are many drawbacks and limitations to the methods based on nucleic acid sequences. For example, PCR efficiency is limited by factors such as DNA length, *secondary structure*, and primer length and design.[4] Unlike PCR, the methods of the present invention do not require the use of primers (*i.e.*, sequence-recognition elements are not used) but instead work with secondary structure (*i.e.*, folded structures) of the sample nucleic acids. In contrast, PCR requires the use of primers, and secondary structure in the sample nucleic acid is detrimental to the reaction.

The Examiner argued that "[o]ne of ordinary skill in the art would have been motivated to combine the teachings of Lyamichev *et al.* with Young, Seela and Roling, and Young *et al.* to produce a method that could be used to optimize allele-specific PCR wherein the polymerase is also a single stranded endonuclease which recognized hairpin structures of the single stranded nucleic acid because Lyamichev *et al.* taught that this method could be used to optimize allele-specific PCR."[5] Appellants must respectfully disagree.

The Examiner has alleged one tenuous and minor point of commonality between Lyamichev *et al.* and Young, Seela and Roling, and Young *et al.* (*i.e.*, the one sentence

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[3] *See e.g.*, specification page 15, lines 24-27, stating that "[i]n one embodiment of the present invention, the treating step comprises rendering double-stranded nucleic acid substantially single-stranded, and exposing the single-stranded nucleic acid to conditions such that the single-stranded nucleic acid assumes a secondary or characteristic folded structure."

[4] *See e.g.*, Specification at page 6, lines 11-15.

[5] Final Office Action, page 8.

reference to allele-specific PCR in the Examiner's discussion of Lyamichev *et al.*). Here, Lyamichev *et al.* briefly mention using the primers of allele-specific PCR, which are unpaired at their 3' end when hybridized to one of the alleles, to act as pilot oligonucleotides to direct selective cleavage of unwanted templates. However, Young, Seela and Roling, and Young *et al.* **do not teach or suggest allele-specific PCR**. There is no teaching or suggestion in Young, Seela and Roling, or Young *et al.* of the discrimination between alleles in a PCR reaction. Thus, there is no justification for making this tenuous connection with Lyamichev *et al.*, and there is no motivation to combine Lyamichev *et al.* and Young, Seela and Roling, and Young *et al.*

Even if Young, Seela and Roling, or Young *et al.* related to allele-specific PCR, a point which the Appellants contest, there is no motivation to combine these references with Lyamichev *et al.*, with respect to the presently claimed invention. The present invention **is not** allele-specific PCR and is not related to allele-specific PCR. As discussed above, the Examiner has alleged one tenuous and minor point of commonality between Lyamichev *et al.* and Young, Seela and Roling, and Young *et al.* (*i.e.*, the one sentence reference to allele-specific PCR in the Examiner's discussion of Lyamichev *et al.*) and used this tenuous point to justify combining the references for all purposes (*i.e.*, combining teachings unrelated to allele-specific PCR). Combinations made by identifying minor points of commonality as a means of generally combining the references are improper when the context of the references, taken as a whole, does not suggest or motivate such a combination. Courts have continuously emphasized that the references must be evaluated as a whole, so that their teachings are

applied in the context of their significance to a technician at the time (*i.e.*, a technician without the Appellants' knowledge of the solution that is provided by the invention).[6]

The cited references, as a whole, do not support their combination by the Examiner. In fact, the tenuous point of commonality (*i.e.*, the reference to allele-specific PCR in Lyamichev *et al.*) ***teaches away*** from the present invention. Lyamichev *et al.* discuss using the unpaired primers of allele-specific PCR to act as pilot oligonucleotides to direct selective cleavage of unwanted templates. In contrast to the presently claimed invention, Lyamichev *et al.*, teach primer-directed cleavage (*i.e.*, a sequence-dependent detection method). As described above, the present invention does not require the use of primers (*i.e.*, it is not a sequence-dependent method). Furthermore, the combination of Lyamichev *et al.* and Young, Seela and Roling, and Young *et al.* as related to allele-specific PCR, ***teaches away*** from the presently claimed invention, as primers are not employed for the cleavage of intra-strand structures. Thus, there is no motivation to combine the cited art, nor has the Examiner pointed to a motivation that would apply to the presently claimed invention. In addition, it is impermissible to combine references when the references ***teach away*** from the combination.[7]

Furthermore, the Examiner's combination of Seela and Roling with Lyamichev is inappropriate because Seela and Roling and the state of the art teach away from such a combination. In particular, the Examiner argued that Seela and Roling taught that the use of "nucleotide analogs helped protect the nucleic acids containing them from nuclease digestion, as well as reduce 'read through' problems frequently encountered in polymerase reactions," (Advisory Action, page 7). With respect to protection from nuclease digestion, Seela and

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[6] *See e.g., Interconnect Planning Corp. v. Feil,* 227 USPQ 543, 551, 774 F.2d 1132, 1143 (Fed. Cir. 1985).

[7] *See e.g., In re Grasselli,* 713 F.2d 731, 218 USPQ 769, 779 (Fed. Cir. 1983).

Roling teach that incorporation of nucleotide analogs protects from cleavage with endonucleases, teaching away from use in methods where cleavage is desired (*i.e.*, the methods of the presently claimed invention). With respect to the reduction of read through problems, the state of the art indicated that the nucleotide analogs improved read through by reducing secondary structure of the nucleic acid (*See e.g.,* Barr *et al.*, BioTechniques 4:428 [1986], of which Seela is a co-author, indicating that 7-deaza-2'-deoxyguanosine-5'-triphosphate prevents formation of higher secondary structures [abstract]; attached hereto at Tab C). The presently claimed invention teaches cleavage at secondary structures. Thus, prior to the presently claimed invention, one would not use the teachings of Seela and Roling in cleavage methods targeted at secondary structure because the art teaches that the nucleotide analogs result in the loss of secondary structure. Only with the teachings of the presently claimed invention (*See e.g.*, Example 37) was it found that nucleotide analogs were compatible with the methods of the presently claimed invention.

Because allele-specific PCR teaches away from the present invention and because Young, Seela and Roling, and Young *et al.* do not teach or suggest either allele-specific PCR or the methods of the present invention, and because Seela and Roling teach away from use of nucleotide analogs in cleavage reactions of secondary structure, there is no motivation to combine the cited references, and *prima facie* obviousness has not been established.

II. The Cited References Do Not Teach or Suggest All of the Elements of the Present Invention

Even if the cited references are combined, the combination of the cited references does not teach or suggest all of the elements of the presently claimed invention. Indeed, the

Examiner has conceded that the primary reference, Lyamichev *et al.*, does not teach "a method for identifying strains of microorganisms, . . . that the detected cleavage products may be compared with cleavage products of nucleic acid structures from reference microorganisms, . . . that the nucleic acid may comprise a nucleotide analog, . . . that the PCR may be done with these nucleotide analogs or that the PCR primers were from ribosomal RNA . . . ."[8]

Young, Seela and Roling, and Young *et al.* do not remedy the deficiencies of Lyamichev *et al.*, as these references only teach general methods of PCR, and do not teach methods for characterization of cleaved nucleic acids. Unlike the presently claimed invention Lyamichev, Young, Seela and Roling, and Young *et al.*, alone or in combination, simply do not teach or suggest the **characterization** (*e.g.*, comparing test cleavage products to control cleavage products or characterizing nucleic acid substrate containing microbial gene sequences) of cleaved nucleic acids.

Furthermore, in contrast to the presently claimed invention, Lyamichev teaches the *detection* of nucleic acids by cleavage of one strand in a structure formed by **inter-strand** annealing (*i.e.*, between a target and a primer) rather than *characterization* of nucleic acids by cleavage of **intra-strand** secondary structures (*See*, Declaration of Mary Ann D. Brow, ¶4; submitted with Appellants' Response to Final Office Action and attached hereto at Tab D).[9]

Specifically, Lyamichev teaches the use of a primer for enzyme recognition of cleavage structures for both RNA and DNA substrates. On page 782, middle column, Lyamichev teaches that "[a]n RNA version of the targeted sequence (Fig. 6A, bottom) was

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[8] Final Office Action, page 6.

[9] Discussed in Appellants' Amendment and Response to the Office Action Dated February 17, 1998, and Appellants' Amendment and Response to the Office Action Dated January 6, 1997.

cleaved . . . in a reaction that was dependent on the presence of a pilot oligonucleotide . . . ." Thus, for RNA substrates, the cleavage structure of Lyamichev requires formation by **inter-strand** annealing between a target and a primer rather than the intra-strand secondary structures of the presently claimed invention (*See*, Declaration of Mary Ann D. Brow, ¶4). In a telephonic interview held on October 9, 1998, the Examiner conceded that a primer is required in the methods of Lyamichev, when RNA is the nucleic acid substrate.

For DNA substrates, Lyamichev teaches that a primer is required when cleavage reactions are conducted under optimal buffer conditions (*See*, Declaration of Mary Ann D. Brow, ¶4). Specifically, on page 780, column 1, Lyamichev teaches that "[i]n the presence of primer, the rate of cleavage was optimal at about 50 mM KCl (Fig. 3A) . . . In the absence of primer, the maximum rate was at about 20 mM KCl, and the reaction was almost completely inhibited at 50 mM KCl (Fig. 2C, lane 1, and Fig. 3B); also, the reactions (Fig. 3B) were incubated 15 times longer than the reactions shown in Fig. 3A)." Thus, Lyamichev teaches that optimal cleavage is achieved at 50 mM KCl in the presence of a primer. In the absence of primer under these salt conditions, cleavage is almost completely inhibited. Furthermore, even at non-ideal salt concentrations, reactions conducted in the absence of primer had to be incubated **15 times** longer to achieve a similar degree of cleavage as compared to reactions containing primer.

Lyamichev also teaches that a primer is required to control the location of the cleavage event (*See*, Declaration of Mary Ann D. Brow, ¶5). For example, on page 779, Lyamichev teaches that "[i]n the absence of primer, cleavage occurred at the ends of the substrate duplexes (either the extended or shortened forms) between the first and second base pairs," and "[t]he primer-directed shifting of the site of cleavage suggests that precise orientation of

the 5' nuclease on the substrate is dominated by the interaction of the polymerization domain of DNAP-*Taq* with the primer." Thus, Lyamichev teaches that a primer is required to control the site of the cleavage. Lyamichev teaches that controlling the site of cleavage with a primer is important in the application of these cleavage methods (*See*, Declaration of Mary Ann D. Brow, ¶6). For example, on page 782, Lyamichev teaches that "[a]n understanding of the 5' nuclease activity has several practical consequences. Most exciting is the creation of cleavage reactions that can cut single-stranded polynucleotides in a highly specific manner at **chosen** sequences . . . essentially any sequence can be targeted for cleavage by annealing it to an appropriate pilot oligonucleotide," (emphasis added). On page 782, last column, Lyamichev teaches that the use of primers "greatly increases cleavage efficiency, and reduces the number of unwanted cleavages at regions of secondary structure in the target nucleic acid." Here, Lyamichev explicitly teaches the use of a primer to avoid **unwanted** cleavages at **intra-strand secondary structures** (*See*, Declaration of Mary Ann D. Brow, ¶6).

Thus, Lyamichev clearly teaches the use of a primer for generating cleavage structures, relates only to *detection* of nucleic acids, and ***teaches away*** from the use of intra-strand secondary structures as targets in cleavage reactions for detection of nucleic acids. Regardless of the presence of absence of a primer, there is no teaching or suggestion in Lyamichev or the other cited references relating to the characterization of cleaved nucleic acids. The cited art fails to establish *prima facie* obviousness because the cited references do not teach or suggest all of the elements of the presently claimed invention (*i.e.*, they do not teach methods for characterization of cleaved nucleic acids and do not teach or suggest the cleavage of intra-

strand secondary structures).[10] Thus, Appellants assert that Claims 1, 3-29, and 31-54 are unobvious over the art cited by the Examiner.

Appellants submit that, with due consideration to all these factors discussed above, the patentability of the claims is evident. Appellants believe that the arguments and Declaration provided during the prosecution were not fully considered. This is made clear by the Examiners statement on page 6 of the Advisory Action that "It is also argued that cleavage specificity and efficiency is improved by the instant claimed invention over the teachings of Lyamichev *et al.*" This is a fundamental misunderstanding of Appellants' arguments and of the technologies. Contrary to the Examiner's interpretation of the arguments, Appellants indicated that Lyamichev *et al.* teach that a primer is required for specificity and efficiency in the cleavage reaction taught by Lyamichev *et al.*, and thus, that Lyamichev ***teaches away*** from the presently claimed invention which does not involve a primer. No comparison was made between the specificity and efficiency of the methods of Lyamichev *et al.* and the methods of the presently claimed invention. Indeed, a comparison is not relevant, as the presently claimed invention relates to cleavage of intra-strand folded structures in methods for characterizing cleaved nucleic acids, while Lyamichev relates to the cleavage of interstrand structures involving a primer and a target in detection reactions.

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[10] *See, e.g., Northern Telecom Inc. v. Datapoint Corp.*, 15 USPQ2d 1321, 1323 (Fed. Cir. 1990); and *In re Dow Chemical Co.*, 837 F.2d 469, 5 USPQ2d 1529 (Fed. Cir. 1988).

For the foregoing reasons, it is submitted that the Examiner's rejection of Claims 1, 3-29, and 31-54 was erroneous, and reversal of the rejection is respectfully requested.

Dated: February 22, 1999

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IX. APPENDIX A: CLAIMS INVOLVED IN THE APPEAL

1. (Twice Amended) A method, comprising:

a) providing:

i) an enzymatic cleavage means;

ii) a test nucleic acid substrate containing sequences derived from one or more microorganisms; and

iii) control cleavage products produced by cleavage of a reference nucleic acid derived from a microorganism;

b) treating said test nucleic acid substrate under conditions such that said substrate forms one or more intra-strand secondary structures;

c) reacting said cleavage means with said intra-strand secondary structures so that one or more test cleavage products are produced; and

d) comparing said test cleavage products to said control cleavage products.

3. (Amended) The method of Claim 1, wherein said enzymatic cleavage means is a nuclease.

4. (Amended) The method of Claim 3, wherein said nuclease is selected from the group consisting of Cleavase™ BN nuclease, *Thermus aquaticus* DNA polymerase, *Thermus thermophilus* DNA polymerase, *Escherichia coli* Exo III, and the *Saccharomyces cerevisiae* Rad1/Rad10 complex.

5. (Amended) The method of Claim 1, wherein said test nucleic acid substrate comprises a nucleotide analog.

6. The method of Claim 5, wherein said nucleotide analog is selected from the group comprising 7-deaza-dATP, 7-deaza-dGTP and dUTP.

7. (Amended) The method of Claim 1, wherein said test nucleic acid of step (a) is substantially single-stranded.

8. (Amended) The method of Claim 1, wherein said test nucleic acid is RNA.

9. (Amended) The method of Claim 1, wherein said test nucleic acid is DNA.

10. (Amended) The method of Claim 1, wherein said test nucleic acid of step (a) is double stranded.

11. The method of Claim 10, wherein said treating of step (b) comprises:

i) rendering said double-stranded nucleic acid substantially single-stranded; and

ii) exposing said single-stranded nucleic acid to conditions such that said single-stranded nucleic acid has secondary structure.

12. The method of Claim 11, wherein said double-stranded nucleic acid is rendered substantially single-stranded by increased temperature.

13. The method of Claim 1, further comprising the step of detecting said one or more cleavage products.

14. The method of Claim 1 wherein said microorganism comprises bacteria.

15. The method of Claim 14 wherein said bacteria are selected from the group comprising members of the genera *Campylobacter, Escherichia, Mycobacterium, Salmonella, Shigella* and *Staphylococcus*.

16. The method of Claim 15 wherein said members of the genus *Mycobacterium* comprise strains of multi-drug resistant *Mycobacterium tuberculosis*.

17. The method of Claim 1 wherein said microorganism comprises virus.

18. The method of Claim 17 wherein said virus is selected from the group comprising hepatitis C virus and simian immunodeficiency virus.

19. (Twice Amended) A method for treating nucleic acid, comprising:

a) extracting nucleic acid from a sample suspected of containing one or more microorganisms; and

b) contacting said extracted nucleic acid with an enzymatic cleavage means under conditions such that said extracted nucleic acid forms one or more intra-strand secondary structures, and said cleavage means cleaves said intra-strand secondary structures to produce a plurality of cleavage products.

20. The method of Claim 19, further comprising the step of separating said cleavage products.

21. The method of Claim 19, further comprising the step of detecting said cleavage products.

22. The method of Claim 21, further comprising comparing said detected cleavage products generated from cleavage of said extracted nucleic acid isolated from said sample with separated cleavage products generated by cleavage of nucleic acids derived from one or more reference microorganisms.

23. The method of Claim 19 further comprising the step of isolating a polymorphic locus from said extracted nucleic acid after the extraction of step a), to generate a nucleic acid substrate wherein said substrate is contacted with the cleavage means of step b.

24. The method of Claim 23 wherein said isolation of a polymorphic locus is accomplished by polymerase chain reaction amplification.

25. The method of Claim 24, wherein said polymerase chain reaction is conducted in the presence of a nucleotide analog.

26. The method of Claim 25, wherein said nucleotide analog is selected from the group comprising 7-deaza-dATP, 7-deaza-dGTP and dUTP.

27. The method of Claim 24 wherein said polymerase chain reaction amplification employs oligonucleotide primers matching or complementary to consensus gene sequences derived from said polymorphic locus.

28. The method of Claim 23 wherein said polymorphic locus comprises a ribosomal RNA gene.

29. The method of Claim 28, wherein said ribosomal RNA gene is a 16S ribosomal RNA gene.

31. (Amended) The method of Claim 19, wherein said enzymatic cleavage means is a nuclease.

32. (Amended) The method of Claim 31, wherein said nuclease is selected from the group consisting of Cleavase™ BN nuclease, *Thermus aquaticus* DNA polymerase, *Thermus thermophilus* DNA polymerase, *Escherichia coli* Exo III, and the *Saccharomyces cerevisiae* Rad1/Rad10 complex.

33. The method of Claim 19, wherein said nucleic acid of step (a) is substantially single-stranded.

34. The method of Claim 19, wherein said nucleic acid is RNA.

35. The method of Claim 19, wherein said nucleic acid is DNA.

36. The method of Claim 19, wherein said nucleic acid of step (a) is double stranded.

37. The method of Claim 36, wherein said treating of step (b) comprises:

i) rendering said double-stranded nucleic acid substantially single-stranded; and

ii) exposing said single-stranded nucleic acid to conditions such that said single-stranded nucleic acid has secondary structure.

38. The method of Claim 37, wherein said double-stranded nucleic acid is rendered substantially single-stranded by increased temperature.

39. The method of Claim 19 wherein said microorganism comprises bacteria.

40. The method of Claim 39 wherein said bacteria are selected from the group comprising members of the genera *Campylobacter, Escherichia, Mycobacterium, Salmonella, Shigella* and *Staphylococcus*.

41. The method of Claim 40 wherein said members of the genus *Mycobacterium* comprise strains of multi-drug resistant *Mycobacterium tuberculosis*.

42. The method of Claim 19 wherein said microorganism comprises virus.

43. The method of Claim 42 wherein said virus is selected from the group comprising hepatitis C virus and simian immunodeficiency virus.

44. (Twice Amended) A method, comprising:

a) providing:

i) an enzymatic cleavage means in a solution comprising manganese; and

ii) nucleic acid substrate containing microbial gene sequences;

b) treating said nucleic acid substrate with increased temperature under conditions such that substantially single-stranded substrate is produced;

c) reducing said temperature under conditions such that said single-stranded substrate forms one or more intra-strand secondary structures;

d) reacting said enzymatic cleavage means with said intra-strand secondary structures so that one or more cleavage products are produced; and

e) detecting said one or more cleavage products.

45. The method of Claim 44, wherein said enzymatic cleavage means is a nuclease.

46. The method of Claim 45, wherein said nuclease is selected from the group consisting of Cleavase™ BN enzyme, *Thermus aquaticus* DNA polymerase, *Thermus thermophilus* DNA polymerase, *Escherichia coli* Exo III, and the *Saccharomyces cerevisiae* Rad1/Rad10 complex.

47. The method of Claim 44, wherein said nucleic acid substrate comprises a nucleotide analog.

48. The method of Claim 47, wherein said nucleotide analog is selected from the group comprising 7-deaza-dATP, 7-deaza-dGTP and dUTP.

49. The method of Claim 44, wherein said nucleic acid is selected from the group consisting of RNA, double stranded DNA and single stranded DNA.

50. The method of Claim 44, wherein said microorganism comprises bacteria.

51. The method of Claim 50, wherein said bacteria are selected from the group comprising members of the genera *Campylobacter, Escherichia, Mycobacterium, Salmonella, Shigella* and *Staphylococcus*.

52. The method of Claim 51, wherein said members of the genus *Mycobacterium* comprise strains of multi-drug resistant *Mycobacterium tuberculosis*.

53. The method of Claim 44, wherein said microorganism comprises virus.

54. The method of Claim 53, wherein said virus is selected from the group comprising hepatitis C virus and simian immunodeficiency virus.

IN THE CLAIMS

1. (Twice Amended) A method, comprising:

a) providing:

i) an enzymatic cleavage means;

ii) a test nucleic acid substrate containing sequences derived from one or more microorganisms [microorganism]; and

iii) control cleavage products produced by cleavage of a reference nucleic acid [sequence] derived from a microorganism;

b) treating said test nucleic acid substrate under conditions such that said substrate forms one or more intra-strand secondary [cleavage] structures;

c) reacting said cleavage means with said intra-strand secondary [cleavage] structures so that one or more test cleavage products are produced; and

d) comparing said test cleavage products to said control cleavage products.

5. (Amended) The method of Claim 1, wherein said test nucleic acid substrate comprises a nucleotide analog.

7. (Amended) The method of Claim 1, wherein said test nucleic acid of step (a) is substantially single-stranded.

8. (Amended) The method of Claim 1, wherein said test nucleic acid is RNA.

9. (Amended) The method of Claim 1, wherein said test nucleic acid is DNA.

10. (Amended) The method of Claim 1, wherein said test nucleic acid of step (a) is double stranded.

19. (Twice Amended) A method for treating nucleic acid, comprising:

a) extracting nucleic acid from a sample suspected of containing one or more microorganisms; and

b) contacting said extracted nucleic acid with an enzymatic cleavage means under conditions such that said extracted nucleic acid forms one or more intra-strand secondary structures, and said cleavage means cleaves said intra-strand secondary structures to produce a plurality of cleavage products.

44. (Twice Amended) A method, comprising:

a) providing:

i) an enzymatic cleavage means in a solution comprising manganese; and

ii) nucleic acid substrate containing microbial gene sequences;

b) treating said nucleic acid substrate with increased temperature under conditions such that substantially single-stranded substrate is produced [said substrate is substantially single-stranded];

c) reducing said temperature under conditions such that said single-stranded substrate forms one or more intra-strand secondary [cleavage] structures;

d) reacting said enzymatic cleavage means with said intra-strand secondary [cleavage] structures so that one or more cleavage products are produced; and

e) detecting said one or more cleavage products.

REMARKS

Claims 1-44 were filed in the original application. Claims 45-54 were added and Claims 2 and 30 were canceled without prejudice in an amendment mailed June 6, 1997.

In the Office Action of August 31, 1998, the Examiner rejected Claims 1, 3-29 and 31-54 under 35 U.S.C. §103(a) as being allegedly obvious under Lyamichev *et al.*, in view of Young, Seela and Roling, and Young *et al.*

Research Report

7-Deaza-2'-Deoxyguanosine-5'-Triphosphate: Enhanced Resolution in M13 Dideoxy Sequencing

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ABSTRACT

Substitution of dGTP with its isosteric analog 7-deaza-2'-deoxyguanosine-5'-triphosphate (c[7]dGTP) allows increased resolution during the M13 dideoxy sequencing of particularly G-C rich regions of DNA. We compared c[7]dGTP with dGTP and 2'-deoxyinosine-5'-triphosphate (dITP), the dGTP analog most frequently used for destabilization of secondary structure during gel electrophoresis. Results of this comparison showed that c[7]dGTP gave enhanced resolution over dGTP and also allowed for increased legibility over longer regions of sequence than with dITP. We ascribe these improved features to an inability of c[7]dG to form higher secondary structures in the polymeric configuration, together with a higher stability of c[7]dG:dC base pairs over those of dI:dC.

INTRODUCTION

Advances in the sequencing of DNA in recent years have allowed the development of procedures for the rapid analysis of large DNA structures. Using M13 dideoxy sequencing methodologies the entire 170 kb Epstein-Barr viral structure (3) and the human mitochondrial genome (2) have been determined. Similarly, the rapidity of these methods has allowed total sequencing of DNA structures from such large genomes as those of the Retroviridae for comparison of strain variation at the DNA level (17, 18, 22, 29). Currently, it is possible to read over 500 bp of DNA sequence in a single reaction using available methodologies. Sequencing technology has also been aided by advances in oligonucleotide synthesis and consequent ease of production of specific primers for sequencing of individual M13 or plasmid templates. However, despite these advances, problems associated with the lack of fidelity in the reading of sequencing gels may occur for one of several reasons. Complementary DNA (cDNA) clones inserted into M13 sequencing vectors may contain long polypurine and/or polypyrimidine tracts depending on the cloning methodology used (6, 15). Sequencing through these regions by the dideoxy method invariably presents difficulties. Particularly G-C rich DNA sequences, exemplified by the recently cloned viral genome for herpes simplex virus type-I (24), demonstrate particularly unfavorable qualities for M13 sequencing. Finally, even in cloned DNA sequences with no apparent abnormality in base composition, band compressions may occur (12). This manifests itself as the product of fragments of different lengths migrating at about the same rate, giving rise to inconclusive or error-prone reading of sequencing gels.

Abnormal gel migration of certain sequences has been presumed to be related to the ability of guanosine residues to form higher secondary structures or "hairpins" during electrophoresis (12). This may be a consequence of Hoogsteen base-pairing involving both N-7 and the exocyclic NH_2 group at the 2 position of the heterocycle (19). Thus, the method of choice for the structure-independent analysis of G-C rich DNA sequence has involved minimizing such interactions by using deoxyinosine-5'-triphosphate (dITP) in place of dGTP for chain extension (12). This removal of the exocyclic amino group, however, considerably weakens hydrogen bonding with C residues, as demonstrated for poly(dI) poly(dC) complexes (8).

Other naturally occurring purine analogs include the pyrrolo [2,3-d] pyrimidines or 7-deazapurine derivatives. For example, 7-deazaadenosine or tubercidin, a potent antitumor agent, is isolated from the culture filtrates of microorganisms (25). In addition, analogs of 7-deazaguanosine such as guanosine are found in the wobble position of certain tRNAs of both prokaryotes and eukaryotes (13). Several reports have described interactions of 7-deazapurine polymers with the corresponding polypyrimidine chains (21, 28). In this report we describe the advantages of using c[7]dGTP, the 7-deaza analog of dGTP (Figure 1), for resolution of abnormal and compressed regions in the dideoxy sequencing of cloned DNA. 7-Deazaguanine, by virtue of replacement of N-7 of the guanine ring by the methine moeity, precludes Hoogsteen bond formation (21). In contrast to inosine, however, Watson-Crick base pairing through the exocyclic amino group at the 2 position of the heterocycle is not impaired (21).

MATERIALS AND METHODS

2'-Deoxy and 2',3'-dideoxynucleoside-5'-triphosphates (dNTPs and ddNTPs) were obtained from P.L. Biochemicals. Sequencing reactions contained 8.7 mM Tris-HCl, pH 7.5, 5.4 mM $MgCl_2$, 72 mM NaCl, 1.5 mM dithiothreitol, plus specific concentrations of nucleoside triphosphate in each of the four base specific reactions. The ddGTP reaction contained 0.034 mM of dATP and dTTP, with 0.0034 mM dGTP and 0.09 mM ddGTP. The ddATP and ddTTP containing reactions followed a similar pattern with the corresponding deoxynucleotide at a ten-fold lower concentration. The ddATP and ddTTP concentrations were .16 mM and .14 mM respectively. The ddCTP-containing reaction was 0.024 mM in each of dGTP, dATP and dTTP, and 0.028 mM in ddCTP. In reactions containing the analogs, dITP or c'dGTP, the concentrations were four-fold greater or equal to the dGTP they replaced, respectively. The dITP-containing reaction also contained 0.109 mM ddITP. Besides the above concentrations of reagents, each reaction contained 0.1-0.5 μg of single stranded M13 DNA from a particular recombinant DNA clone, a synthetic deoxyoligonucleotide primer (0.04-0.05 μM), and 5.6 μCi of either 2'-deoxycytidine 5'-[α-^{32}P]-triphosphate (410 Ci/mmole) or 2'-deoxycytidine 5'-([α^{35}S]thio)-triphosphate (410 Ci/mmole) (Amersham). Deoxyoligonucleotide primers were synthesized by the phosphoramidite method using Applied Biosystems 380A DNA synthesizers. The reactions were initiated by addition of 0.5 units of DNA polymerase I, large fragment (Boehringer/Mannheim or Pharmacia), chased after 20 min at room temperature with addition of all four deoxynucleotides to a concentration of 0.2 mM and then incubated for 15 min. The reactions were then terminated by addition of two volumes of 95% formamide (MCB) containing 0.05% each of bromophenol blue and xylene cyanol (Sigma). Samples were incubated for 2 min at 95°C and allowed to cool immediately prior to electrophoresis. The reactions with the dGTP analogs were as stable as the normal deoxynucleotides and could be stored at -20°C for up to 24 h with little or no loss in resolution.

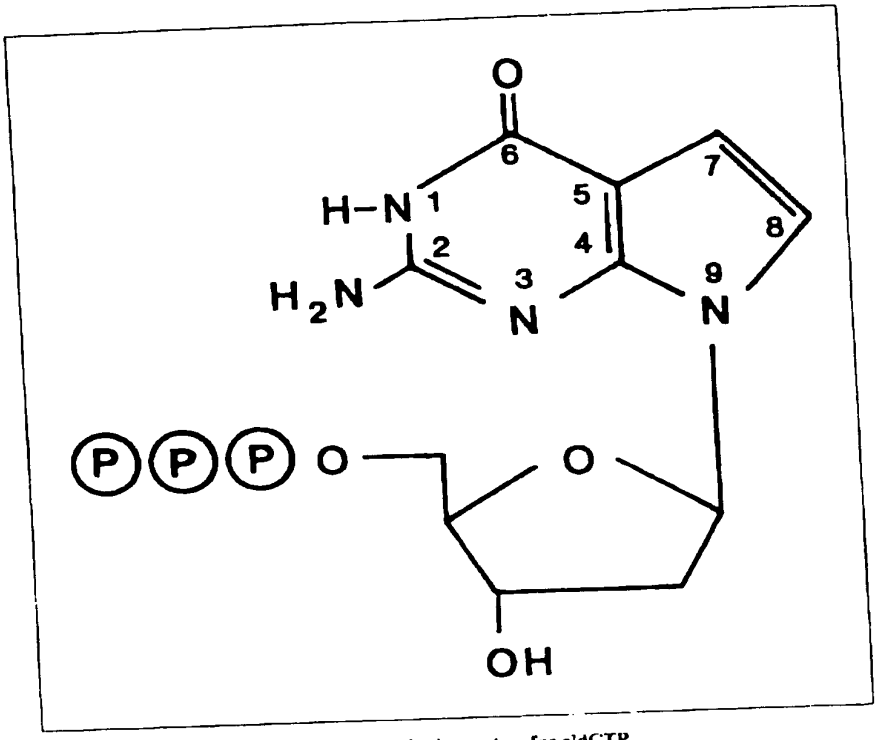

Figure 1. Chemical structure and ring numbering system for c'dGTP.

Synthesis of c'dGTP

The chemical synthesis of c'dGTP has been described previously (20).

Briefly, 7-deaza-2'-deoxyguanosine was prepared by phase transfer glycosylation of 2-amino-4-methoxy-7H-pyrrolo [2,3-d] pyrimidine with 1-chloro-2-deoxy-3,5-di-O-p-toluoyl-D-*erythro*-pentofuranose followed by removal of protecting groups. Monophosphorylation with phosphorus oxychloride in trimethyl phosphate followed by treatment with 1,1'-carbonyldiimidazole and bis-triethylammonium pyrophosphate gave c'dGTP as its triethylammonium salt.

Electrophoresis

Acrylamide and N,N'-methylenebisacrylamide were purchased from BRL, and mixed in a 24:1 ratio to give a 40% acrylamide stock solution. 23 x 8 inches 5% acrylamide gels of 0.015 inches in thickness were run as follows: Aliquots of 2 μl from dideoxy sequencing procedures were loaded on two separate Tris-borate/urea/polyacrylamide gels (11, 16) and electrophoresed for 3 h at 1800 V (short gel) and 17 h at 1350 V (long gel). Gels were dried under vacuum and autoradiographed for 4-12 h.

RESULTS AND DISCUSSION

We have compared dGTP, dITP and c'dGTP in the sequencing of particularly G-C rich DNA. Figure 2A shows one such region of the herpes simplex virus (HSV) type-I genome (24). This example demonstrates a typical compressed region in the G-track [lane (a)], giving rise to indecipherable banding patterns and consequent ambiguity in sequence assignment. Correction of this ambiguity could be achieved using dITP in place of dGTP [lane (b)], however

in this region of the gel it is apparent that bands in all tracks have lost intensity, again giving potential sequence ambiguity. A quantitative comparison of this analog with dGTP was assessed using data derived from many sequencing reactions. Use of dGTP in our procedures (>1000 reactions) accurately assigns over 500 bp in a single sequence reaction using separate "long" and "short" gels (see experimental procedures). With dITP, because of this decrease in band intensity the length of accurate sequence determination is reduced to around 150-300 bp (data derived from >300 sequence reactions). Lane (c) shows the highly increased resolution of the compressed region using c⁷dGTP in place of dGTP. Furthermore, it is apparent that the signal intensity provided by this analog is equal to that using dGTP. Quantitative assessment of additional (>200) sequencing reactions using c⁷dGTP showed that this analog was equal to dGTP in terms of sequence length assignable. Again, over 500 bp of DNA sequence could be read with this analog in each sequence reaction.

In the extreme case of G-C rich DNA, namely poly(dG) or poly(dC) tails produced from cDNA cloning, c⁷dGTP was unable to resolve DNA sequence efficiently (data not shown). This is analogous to observations with dGTP and dITP and adds weight to the notion that difficulties associated with reading poly(dG) or poly(dC) tails are not related to secondary structures formed during electrophoresis. A possible explanation for band heterogeneity in these cases could be that of frameshifting during template copying. This could arise either in the sequencing reaction or through heterogeneous template production during replication. It is known that homopolymeric regions are more prone to certain types of frameshifting phenomena (30). For example, translational frameshifting within dG tails of cDNA expression libraries hasbeen observed recently (31).

Further demonstrations of the efficacy of c⁷dGTP are shown in figures 2(B)-(D). During sequencing of DNA from the transposon TN5 neomycin phosphotransferase gene (5), a compression in the C-track could be efficiently resolved using c⁷dGTP (Figure 2B, arrowed). Figure 2C shows the results of sequencing a particularly G-C rich region of a synthetic gene for bovine basic fibroblast growth factor

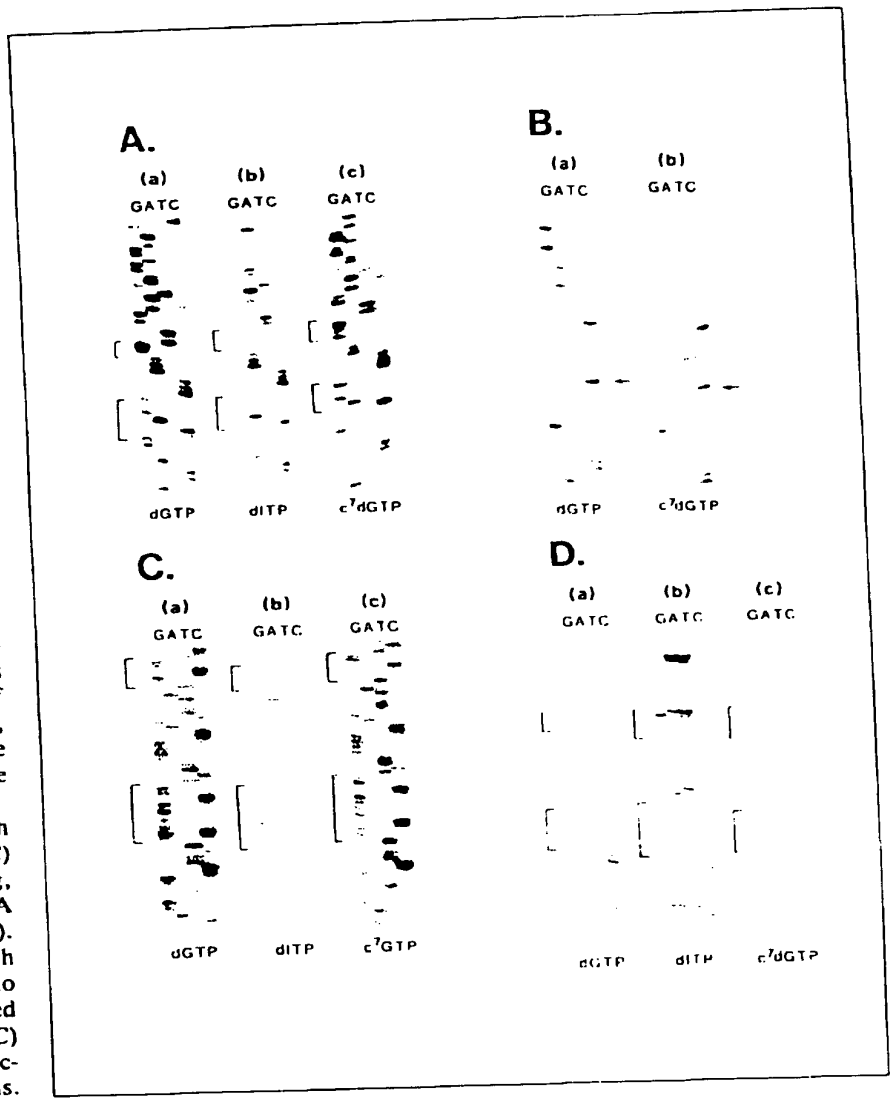

Figure 2. Comparative examples of selected regions of DNA sequence using dGTP, dITP and c⁷dGTP in M13 dideoxy sequencing. (A) A band compression region using dGTP [lane (a)] in the sequence GGGG from HSV-I and its resolution using dITP [lane (b)] and c⁷dGTP [lane (c)]. The lower parentheses show a region (5'-CCAGCG-3') which was difficult to assign using dITP [lane (b)] by virtue of lack of intensity for a given exposure time. With the same exposure time, however, c⁷dGTP resolved both this region and the compressed region [lane (c)]. (B) Resolution of a compression in the C-track of the sequence 5'-TGCCTG-3' of the transposon TN5 neomycin phosphotransferase gene using dGTP [lane (a)] and c⁷dGTP [lane (b)], arrowed. (C) Sequence assignment of a particularly G-C rich region of a synthetic bbFGF gene. Lower parentheses enclose a compression in the synthetic sequence 5'-GGCCGGGGCCGG-3' using dGTP [lane (a)]. Although partially resolved using dITP [lane (b)], further ambiguities arise (upper parentheses). Both regions are fully resolved with c⁷dGTP [lane (c)]. (D) Using α³⁵S-dCTP the regions 5'-CGGCCGC-3' (upper parentheses) and 5'-TGCCGCTG-3' (lower parentheses) are unresolved with either dGTP [lane (a)] or dITP [lane (b)], c⁷dGTP however, permits accurate sequence assignment [lane (c)].

(bbFGF) (7). With dGTP [lane (a)] several compressions and "ghost" bands are visible. dITP, although improving the compressed regions [lane (b)] could not fully resolve this region of DNA sequence and again lack of band intensity is apparent. c'dGTP however [lane (c)], gave clearly legible sequence throughout the entire region. Additional confirmation of the sequence of the synthetic bbFGF gene was provided by sequencing of the complementary strand, and also by expression of a biologically active bbFGF polypeptide in *E. coli* and yeast (4). Finally, in another region of the TN5 transposon neomycin phosphotransferase gene (5) where sequence was completely illegible with both dGTP and dITP [Figure 2D, lanes (a) and (b)], c'dGTP was able to resolve the region effectively [lane (c)]. Again, this sequence was verified by analysis of the complementary strand. This particular example also extends the versatility of c'dGTP to sequencing using α"S-dCTP as the label.

In addition to the examples shown, c'dGTP has made possible the unambiguous sequence determination of large segments of the herpes simplex virus types I and II genomes (24), complete rabbit and human aldolase B genes (1, 27), a corn aldolase cDNA (9) and regions of the *E. coli* chromosome (14). Together, these examples show the generality and efficiency of c'dGTP in the dideoxy method of DNA sequencing. We ascribe these features to the base pairing properties of c'dGTP. First, it has been shown that poly (c'G):poly(C) has an almost identical Tm (75 °) to poly(G):poly(C) (74 °) in 0.2 mM EDTA at pH 5.3 (21). This data implies that the stereochemical arrangement of c'G in a base-paired Watson-Crick double helix is very similar to that of G, and would be analogous in the case of the corresponding polydeoxyribonucleotides. This most probably explains the high correlation in chain extension attainable with c'dGTP and dGTP as observed on sequencing gels. In contrast, dITP with no exocyclic NH_2 at the 2-position, cannot base pair in a full three-hyd.ogen bond Watson-Crick configuration and is also a relatively poor substrate for DNA polymerase, giving possible explanations for the decreased length of sequence which can be read using this analog. Similarly, although inosine containing polymers can form higher secondary structures through Hoogsteen base pairing (26), again this cannot involve three hydrogen bonds as with guanosine containing polymers (19). Consequently, we would propose that this, and the observation that removal of nitrogen at the 7-position in 7-deazaguanine containing polymers precludes completely Hoogsteen bond formation (21), are the most likely explanations for the favorable gel electrophoresis properties of polymers containing these analogs. Thus, the combination of stable Watson-Crick base pairing capability and the inability to form higher secondary structures make c'dGTP an ideal reagent for the structure independent resolution of DNA sequence during the dideoxy method of sequence analysis.

Recent advances in automated sequencing processes have indicated the potential for computer assisted gel reading and data entry (10, 23). We believe that analogs such as c'dGTP will be essential for the accurate automation of DNA sequence gel reading in addition to their usefulness in the manual procedures currently available.□

While this work was in progress, Mizusawa *et al.*, Nucleic Acids Res. *14*, 1319-1324, 1986) reported similar improvements in DNA sequencing using c'dGTP.

Addendum: c'dGTP and sequencing kits containing this analog may be obtained from Boehringer-Mannheim (Indianapolis, IN 46250) or from American Bionetics (Emeryville, CA 94608).

ACKNOWLEDGMENTS

We thank Drs. Rae Lyn Burke and Barbara Chapman for DNA sequences for use as examples and Toni H. Jones and Diana M. Wyles for preparation of the manuscript.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Brow *et al.*
Serial No.: 08/520,946
Filed: 08/30/95
Entitled: **Rapid Detection and Identification of Pathogens**

Group No.: 1636
Examiner: W. Sandals

DECLARATION OF MARY ANN D. BROW UNDER 37 CFR §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: **Box Non-Fee Amendment**, Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: November 2, 1998

By: Kamrin T. MacKnight

1. I, Mary Ann D. Brow, am one of the joint inventors of the subject matter embodied in the above-identified patent application.

2. I am familiar with the Final Office Action from the Patent Office mailed August 31, 1998 in the above-named application (*i.e.*, U.S. Patent Appln. Ser. No. 08/520,946).

3. In the Final Office Action, the Examiner rejected Claims 1, 3-29 and 31-54 as being allegedly obvious under Lyamichev *et al.*, in view of Young, Seela and Roling, and Young *et al.* I am a co-author of Lyamichev *et al.* and contributed to and am familiar with the experimental data in Lyamichev *et al.*

4. The experimental findings in Lyamichev *et al.* teach away from the use of intra-strand secondary structures as targets for cleavage means. Instead, the experimental findings in Lyamichev *et al.* teach the use of cleavage structures formed by inter-strand annealing between a primer and a target nucleic acid. In the absence of primer, target nucleic acid molecules were cleaved poorly, or not at all. Specifically, as described on page 782 of Lyamichev *et al.*, we showed that cleavage of RNA substrates was dependent on the presence

of a primer. Additionally, as described on page 780, column 1 of Lyamichev *et al.*, we obtained optimal cleavage of DNA substrate in 50 mM KCl in the presence of a primer. In the absence of primer at this optimal KCl concentration, cleavage was almost completely undetectable (See Figure 3B). When buffer conditions were optimized for cleavage without primer, reactions had to be incubated for approximately fifteen times longer to obtain a similar degree of cleavage as compared to reactions containing primer.

5. The experimental findings in Lyamichev *et al.* also demonstrate that a primer is required to control the location of the cleavage event. For example, as described on page 779 of Lyamichev *et al.*, we showed that, in the absence of primer, cleavage occurred at the ends of the substrate duplexes between the first and second base pairs and that the primer-directed shifting of the site of cleavage suggested that precise orientation of the 5' nuclease on the substrate was dominated by the interaction of the polymerization domain of DNAP-*Taq* with the primer.

6. Lyamichev *et al.* further teaches that a primer is important for applications using the cleavage reactions. On page 782 of Lyamichev *et al.*, we explain that the use of primers greatly increases cleavage efficiency, and reduces the number of unwanted cleavages at regions of secondary structure in the target nucleic acid. Without primer, we found that the location of the cleavage was unpredictable, undesired cleavage was caused by exogenous DNA (contaminant DNA), and undesired cleavage occurred at regions of secondary structure in the target nucleic acid. Thus, in Lyamichev *et al.*, we taught that primers were needed for applications using the cleavage reactions and that cleavage of intra-strand secondary structures as targets in cleavage reactions was undesired.

7. The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Dated: Nov 1, 1998 Signed: [signature]
Mary Ann D. Brow